Molecular Ecology Notes (2005) **5**, 791–793 doi: 10.1111/j.1471-8286.2005.01064.x

PRIMER NOTE

Isolation and characterization of eight microsatellite loci from *Phaedranassa tunguraguae* (Amaryllidaceae)

NORA H. OLEAS,* ALAN W. MEEROW† and JAVIER FRANCISCO-ORTEGA*
*Florida International University, Biology Department, 11200 SW 8th St., Miami, Florida, 33199, USA, †USDA-ARS-SHRS, National Germplasm Repository, 13601 Old Cultler Road, Miami, Florida 33158, USA

Abstract

***Phaedranassa tunguraguae* is an endangered species endemic to Ecuador. Eight highly polymorphic microsatellite loci were isolated from an enriched genomic library for this species. Levels of polymorphism were evaluated using a total of 31 individuals from a single natural population. An average of 14.1 alleles per locus was detected, and observed heterozygosity ranged from 0.387 to 0.903. All but one locus depart significantly from Hardy–Weinberg equilibrium. These loci are the first microsatellite primers isolated for Amaryllidaceae and will be utilized to investigate patterns of genetic variation of *P. tunguraguae*, which will contribute data relevant to the conservation of the species.**

Keywords: Amaryllidaceae, conservation, microsatellite, *Phaedranassa*, population genetics, SSR

Received 01 April 2005; revision accepted 28 April 2005

Phaedranassa Herb. is a small genus which has been recognized as a monophyletic group in the Andean clade of the Amaryllidaceae (Meerow *et al*. 2000). This Neotropical genus includes nine species, six of them restricted to Ecuador (Meerow 1990). The geographical distribution of the Ecuadorian species is limited to dry valleys and wet slopes of the northeast Andes (Meerow 1990; Oleas 2000). *Phaedranassa tunguraguae* Rav. is endemic to Ecuador. Five patchy populations of this species were located in an area of approximately 15 km^2, in the Pastaza river valley and in the eastern slopes of the Tungurahua Volcano above Baños (Meerow 1990; Oleas 2000). The populations of this species are apparently isolated and occur in one of the most deforested regions of Ecuador, where almost nothing of the native vegetation is remaining (Valencia *et al*. 1999). As a result, *P. tunguraguae* is listed as endangered under the latest IUCN's criteria (Oleas 2000).

A microsatellite enriched library was built following a method modified from Edwards *et al*. (1996) and explained in detail in Alghanim & Almirall (2003). Genomic DNA was extracted from one fresh leaf of *P. tunguraguae* using a modified Dellaporta *et al*. protocol (1983), then digested, linked with adaptors and amplified with polymerase chain reaction (PCR). The amplicons were enriched twice with biotin-labelled oligoprobes and then separated with streptavidin-coated magnetic beads (Dynal). Following the enrichment, products were size separated with a Sepharose CL-4B SizeSep 400 Spun Column (Amersham Pharmacia Biotech), cloned using a TOPO TA Cloning Kit (Invitrogen Corp.) and screened by sequencing.

We obtained 516 colonies, 135 of them containing microsatellite repeats (26%). The sequences were analysed with DNA SEQUENCING ANALYSIS software version 3.7 (Applied Biosystems). The fragments containing microsatellite repeats were imported to SEQUENCHER version 4.1 (Gene Codes Corp.) to clean up and generate consensus sequences.

The majority of the repeats were dinucleotide (70 sequences) followed by trinucleotide (30 sequences) and complex (30 sequences). The most frequent dinucleotide repeats corresponded to the combination AG/TC (84%), followed by AC/TG (26%). Six trinucleotide repeats were found: AAG/TTG (47%), AAC/TTG (23%), ATC/TAG (13%), AGG/TCC (7%), ATG/TAC (7%) and CTG/GAC (3%). Three sequences contained pentanucleotide repeats (two ATATG/TATAC and one ATATC/TATAG), and one sequence contained a hexanucleotide (AAAAAG/TTTTTC).

Only 95 sequences were suitable for primer design (16%) and 10 of them were redundant. PCR primer sequences were designed from the flanking regions of the

Correspondence: N. Oleas, Fax: 305 969 6410; E-mail: nolea002@fiu.edu

Table 1 Primer sequences and basic descriptive statistics of eight microsatellite loci from *Phaedranassa tunguraguae*

Locus	GenBank Accession no.	Primer sequences (5′–3′)	Repeat	*n*	No. of alleles	Allele size range (bp)	H_O	H_E
Pt4	AY994159	F: TCCTTGTATCGTATGCTCCC R: CAAACGCTGTATCCCCTTC	$(CT)_{23}$	30	16	105–250	0.323	0.912*
Pt9	AY971877	F: AAAACCCTAAGGAGAGAGGAG R: GAAATTTGACGATGAACGGAC	$(GA)_{17}$	31	16	87–125	0.710	0.939*
Pt12	AY971878	F: ACAATCACAAACACGGACAC R: AGAACCTACACCCTCTATTGAC	$(GAT)_7$	29	6	329–344	0.387	0.617*
Pt14	AY971879	F: GGAGGATGGTAGTACCATGAAC R: TGTATGGTTGGGTATGGGAAC	$(GA)_{14}$	31	17	153–191	0.903	0.886
Pt21	AY971880	F: GATGGAAAATGATGACGCAAG R: ACCCTTCCTTTGAACCCTAC	$(GT)_9(GA)_9$	30	15	146–184	0.774	0.893*
Pt32	AY934527	F: AACGTACACGTACATTGTCTG R: TCCTTGATCACGAAAATCGTAG	$(CT)_{18}$	29	11	178–199	0.613	0.863*
Pt36	AY971881	F: AGAGAATGTGATGGGAGAGAG R: TCTTCCTTATCCCCTCCACC	$(GA)_{22}$	29	21	163–246	0.613	0.947*
Pt39	AY971882	F: TCAAAACACTCATACCAACACC R: CCTCTCTCTCCAAACTCTCTC	$(CA)_{10}$	28	11	232–264	0.548	0.733*
Mean					14.1		0.608	0.849

H_O, observed heterozygosity; H_E, expected heterozygosity. *Departs high significantly from HWE at $P < 0.01$.

85 unique sequences using GCG primer design protocol (Accelrys).

A total of 40 primers were tested in eight DNA individuals by PCR with fluorescent dUTP (Applied Biosystems). Only 20 of them amplified and captured polymorphism in more than four individuals. Fluorescently labelled forward primers were obtained from these 20 and used to amplify DNA of 31 individuals collected in a natural population in Baños, Ecuador.

The 10-μL reaction mix consisted of 1 μL template DNA (*c.* 20 ng/μL), 6.25 μL dH_2O, 1 μL 10× buffer with 15 mM $MgCl_2$, 0.25 μL 10 mM dNTP, 0.05 μL Ampli*Taq* DNA polymerase (5U/μL) (Applied Biosystems), 0.25 μL fluorescent 5′ end-labelled forward primer (10 μM) and 0.25 μL reverse primer (10 μM). The 31 DNA samples were PCR amplified with the following touchdown program: 5 min at 94 °C; 45 s at 94 °C; 45 s at 60 °C; 60 s at 72 °C; steps 2 repeated 14 times (< 1 degree/cycle); 45 s at 46 °C; 60 s at 72 °C; steps 6 though 8 repeated 25 times and 4 °C storage. The products were loaded in an ABI 3730 Genetic Analyser (Applied Biosystems) and allele size were identified by capillary gel electrophoresis using GeneScan 500 ROX size standard (Applied Biosystems). Raw data were imported into GENEMAPPER 3.0 (Applied Biosystems) for allele calls. Descriptive statistics (Table 1) were generated with MSA 3.15 (Dieringer & Schlotterer 2003). Exact tests for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were run with GENEPOP 3.4 (Raymond & Rousset 1995).

Eight primer pairs were highly polymorphic (Table 1). An average of 14.1 alleles per locus was detected, and observed heterozygosity ranged from 0.387 to 0.903. With the exception of Pt14, all the loci departed significantly from HWE in our test population. None of the loci showed LD ($P < 0.005$).

The high levels of polymorphism of these loci make them useful for the investigation of genetic variation in *P. tunguraguae*, and will be useful for refining conservation efforts for this species.

Acknowledgements

The authors gratefully acknowledge financial support from NSF Grant DEB 0129179, and Jason R. Clayton for technical assistance. We also thank Herbario QCA and Ministerio del Medio Ambiente of Ecuador for collection and export permits (004-IC-INEFAN-DNANUS-US). This is contribution no. 88 of the Tropical Biology Program at Florida International University.

References

Alghanim HJ, Almirall JR (2003) Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analyses. *Analytical and Bionalytical Chemistry*, **376**, 1225–1233.

Dellaporta SL, Wood J, Hicks JB (1983) Isolation of DNA from higher plants. *Plant Molecular Biology Reporter*, **4**, 19–21.

Dieringer D, Schlotterer C (2003) MICROSATELLITE ANALYZER (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes*, **10**, 1–3.

Edwards KJ, Barker JHA, Daly A, Jones CJ, Karp A (1996) Microsatellite libraries enriched for several microsatellite sequences in plants. *BioTechniques*, **20**, 758–760.

Meerow AW (1990) Amaryllidaceae. In: *Flora of Ecuador* (eds Harling G, Andersson L), vol. 41. University of Gotenborg; Riksmuseum, Pontificia Universidad Católica del Ecuador, Goteborg, Stockholm, Quito.

Meerow AW, Guy CL, Li Q (2000) Phylogeny of the American Amaryllidaceae based on nrDNA ITS sequences. *Systematic Botany*, **25**, 708–726.

Oleas N (2000) Amaryllidaceae. In: *Libro Rojo de las Plantas Endémicas del Ecuador 2000* (eds Valencia R, Pitman N, Leon-Yanez S, Jørgensen PM), pp. 66–67. Publicaciones del Herbario QCA, Pontificia Universidad Católica del Ecuador, Quito.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact test and ecumenicism. *Journal of Heredity*, **83**, 239.

Valencia R, Cerón C, Palacios W, Sierra R (1999) Las formaciones Naturales de la Sierra del Ecuador. In: *Propuesta Preliminar de un Sistema de Clasificación de Vegetación para el Ecuador Continental* (ed. Sierra R). Proyecto INEFAN/GEF-BIRF y EcoCiencia, Quito.